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Growth Inhibitor and Differentiation Factor MRG

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### **Abstract**

We have previously identified and characterized a novel tumor growth inhibitor and a fatty acid binding protein in human mammary gland and named it as Mammary derived growth inhibitor Related Gene MRG. MRG has tumor-suppressing activities; it inhibits breast cancer cell growth in vitro and tumor growth in vivo. Here, the effects of MRG on mammary gland differentiation and its interaction with  $\omega$ -3 polyunsaturated fatty acids ( $\omega$ -3 PUFA) on growth inhibition were investigated. MRG protein expression was associated with human mammary gland differentiation with the highest expression observed in the differentiated alveolar mammary epithelial cells from the lactating gland. Overexpression of MRG in human breast cancer cells induced differentiation with changes in cellular morphology and a significant increase in the production of lipid droplets. Treatment of mouse mammary gland in organ culture with MRG protein resulted in a differentiated morphology and stimulation of  $\beta$ -casein expression. Treatment of human breast cancer cells with  $\omega$ -3 PUFA DHA resulted in a differential growth inhibition proportional to their MRG expression. MRG transfected cells or MRG protein treated cells were much more sensitive to DHA-induced growth inhibition compared with MRG negative or control non-treated cells. Our results suggest that MRG is a candidate mediator of the differentiating effect of pregnancy on breast epithelial cells and may play a major role in  $\omega$ -3 PUFA-mediated tumor suppression.

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## I. BACKGROUND AND SIGNIFICANCE

MRG was cloned in normal human mammary gland by differential cDNA sequencing aimed at the identification of growth inhibitory factors of the normal mammary gland (1). The sequence of MRG was found to be highly homologous to human heart type FABP (H-FABP) and identical to the recently identified human brain type FABP (B-FABP) (2). FABPs comprise a well-established family of cytoplasmic hydrophobic ligand binding proteins and are thought to be involved in lipid metabolism by binding and transporting long-chain fatty acids intracellularly. However, other studies have implicated different roles for FABPs in cell signaling, growth inhibition, and differentiation. The most characterized biological functions for H-FABP and B-FABP are tumor suppressing activities against breast cancer. These include that 1) the loss of H-FABP/MDGI (3) and B-FABP/MRG expression (1) is associated with breast cancer progression; 2) both MDGI (4-6) and MRG (7) are highly expressed in the fully differentiated lactating mammary gland and induce mammary gland differentiation; 3) MDGI and MRG have been mapped at the chromosome 1p35 (8) and 6q22-23 (2) that harbor the putative tumor suppressor genes for breast cancer (9-10); and 4) both MDGI and MRG strongly suppress the growth of breast tumors (8, 1).

It is well established that  $\omega$ -3 PUFAs, primarily DHA and EPA in fish oil, suppress mammary tumorigenesis *in vivo* and breast cancer cell proliferation *in vitro* (11-15). As a member of FABP, it has been previously reported that  $\omega$ -3 PUFA DHA is the physiological ligand for mouse MRG (B-FABP) based on its high binding affinity ( $K_d$  10nM) (16). We have demonstrated that the gene encoding MRG has a strong tumor suppressor activity (1). The magnitude of the tumor suppressing activity of MRG on mammary tumor is comparable to that previously observed for *Rb* and *p53* (17). In the current study, we investigated the effects of MRG on mammary differentiation and its interaction with DHA on the growth of breast cancer cells. Our data suggest that MRG is a differentiation factor for breast epithelial cells and may play a major role in DHA-mediated growth suppression of breast cancer cells.

## II. WORK ACCOMPLISHED

### Specific Aim 1:

#### 1. Screening of MRG expression in a variety of human breast tissues. Finished (see Fig. 1 in attached paper).

Association of MRG expression with mammary gland lactation. In an attempt to evaluate the potential biological significance of MRG on differentiation and lactation of human mammary gland, we studied MRG protein expression in the formalin-fixed and paraffin-embedded clinical human biopsy specimens from normal breast reduction mammoplasty specimens, lactating mammary glands, and malignant breast carcinomas.

Fig. 1 shows a representative immunohistochemical staining for MRG. The terminally differentiated lactating mammary gland is characterized by ducts branching into distended and large lipid-rich active secretory lobuloalveolar structures. An increase in cell volume due to cytoplasmic vacuolation and the presence of secretory vesicles containing milk proteins was clearly noted in the lactating gland (Fig. 1B). We found a strongly positive MRG protein staining in the alveolar mammary epithelial cells from the lactating mammary gland (Fig. 1C). The expression of MRG protein was clearly detectable in the alveolar epithelial cells in all 5 lactating mammary glands. In contrast, either no detectable MRG protein staining or very weak MRG protein expression was visualized in 8 cases of the non-pregnant normal breast reduction mammoplasty specimens from nulliparous women (Fig. 1D). Expression of MRG protein was absent in all 10 cases of malignant breast carcinomas (Fig. 1E).

#### 2. Effects of MRG on mammary cell differentiation. FINISHED (See Figs. 3-5 and Table 1 in attached paper).

Induction of differentiation of breast cancer cells. To investigate if the high level of MRG expression in the lactating alveolar mammary epithelial is an instigator or merely a by-product during mammary gland differentiation leading to the milk production, we investigated whether overexpression of MRG gene could induce differentiation. We transfected MDA-MB-231 human breast cancer cells with full-length MRG cDNA and established several MRG expressing clones (MRG-231 clones) (1). Fig. 3A shows the MRG protein expression in MRG-231-10 and MRG-231-6 cells, two MRG positive clones, but not in parental MDA-MB-231 and neo-231-1 MRG negative cells.

It is well established that the extracellular matrix is required for normal functional differentiation of mammary epithelia. Striking changes in cell morphology were observed when MRG-231 cells were cultured in the Matrigel coated dish. MRG-231-10 cells were aggregated to form spheroids on a reconstituted basement membrane gel (Fig. 3B), a typical differentiated phenotype for mammary epithelial cells (28). In contrast, neo-231-1 cells showed considerable heterogeneity in cell size, and many cells had "fibroblast-like" spreading morphology (Fig. 3C).

We examined whether MRG-induced morphological changes are consistent with differentiation. Because the maturation of breast cells is characterized by the presence of lipid droplets that are milk components, we examined the lipid accumulation on MRG-231 cells compared with the control cells. Droplets containing neutral lipid were readily detectable in MRG-231-6 clones cultured in the non-coated culture plates; in contrast, no obvious lipid droplet could be observed in the neo-231-1 cells. When the lipid-producing cells were counted, 2 % and 5 % of MRG-231-6 and MRG-231-10 cells produced lipid droplets, respectively, but virtually no lipid producing cells were observed in MDA-MB-231 and neo-231-1 cells. When the cells cultured in the Matrigel-coated

plates, a significant increase in lipid accumulation was observed in both MRG-231 cells and MRG negative control cells. Representatives of lipid staining in MRG-231-6 and neo-231-1 cells were shown in Fig. 4. Fifteen % of MRG-231-6 and 21% of MRG-231-10 cells produced lipid droplets, but only 4 % of MDA-MB-231 cells and 3 % of neo-231-1 contained lipid droplets, which were much smaller size than that of MRG positive cells (Table 1).

Induction of differentiation of mouse mammary gland by MRGp. Tissue-specific expression of milk protein in mammary epithelial cells depends on contact with stromal cells and matrix proteins. To further confirm the differentiating effect of MRG on mammary gland, we used the mouse whole-organ culture of mammary gland to study whether MRGp can regulate milk protein  $\beta$ -casein. The glands from virgin mice were cultured for 6 days with or without 50 nM MRGp. In mammary gland development, the alveolar buds represent a developmental pathway that eventually leads to secretory alveoli during functional differentiation. Histological examination of MRGp-treated glands revealed the appearance of secretory active alveoli with enlarged luminal spaces and the induction of lipid accumulation (Fig. 5, A & B). In consistent with these changes, which are characteristic for the differentiated phenotype, functional differentiation with stimulation of  $\beta$ -casein was also observed. While no detectable  $\beta$ -casein mRNA was observed in control mammary glands, expression of  $\beta$ -casein mRNA was significantly increased in MRGp treated glands (Fig. 5, C & D). Therefore, treatment of mouse mammary gland in organ culture with MRGp resulted in a histologically differentiated phenotype as well as functional differentiation.

**Specific Aim 2:** MMTV/MRG transgenic mice. IN PROGRESS.

**New Studies:** Interaction of  $\omega$ -3 PUFA DHA and MRG on cell growth (See Figs. 6-7 in attached paper).

Since MRG is a fatty acid binding protein with the highest binding affinity to  $\omega$ -3 PUFA DHA, we were interested in studying whether the growth-suppressing effect of DHA is mediated in part by MRG. We first studied the effects of DHA on MRG negative MDA-MB-231 cells. The cells were treated with DHA at the doses of 2, 4, 6, 8, and 12  $\mu$ g/ml for four days with fresh DHA added every two days. A very narrow dose-dependent growth inhibition was observed for DHA (Fig. 6A). While no significant growth inhibition was observed for DHA at the doses of 2  $\mu$ g/ml, 71% and 92% of growth inhibition was observed at the doses of 8 and 12  $\mu$ g/ml, respectively. We therefore chose the non-inhibiting DHA dose of 2  $\mu$ g/ml for testing its growth-regulatory effect on MRG positive vs. MRG negative cells. As demonstrated in Fig. 6B, when the cells were treated with 2  $\mu$ g/ml of DHA, 55% and 47% of growth inhibition were observed in MRG-231-6 and MRG-231-10 MRG transfected cells, respectively. However, no growth inhibition was observed in MRG negative parental MDA-MB-231 cells and neo-231-1 cells. We also studied the effect of  $\omega$ -6 fatty acid linoleic acid on the growth of MDA-MB-231 cells. At the same conditions as we did for  $\omega$ -3 fatty acid DHA, no significant growth effect was observed at the similar dose range between 4 to 20  $\mu$ g/ml (data not shown).

To further confirm the synergistic interaction of MRG expression and DHA on growth inhibition, we treated MRG negative MDA-MB-436 and MDA-MB-468 cells with DHA and MRGp. MRGp treatment induced a dose-dependent growth inhibition in MDA-MB-436 breast cancer cells (Fig. 7A). While no significant growth inhibition was observed when MRGp dose was below 50 nM, 10% and 14% of growth inhibition was observed when cells were treated with 50 nM and 80 nM of MRGp, respectively. At 150 nM of MRGp, the growth was inhibited 58%. A sub-maximal MRG

dose of 80 nM was used to test the interaction between MRG and DHA. Treatment of MDA-MB-436 (Fig. 7B) and MDA-MB-468 (Fig. 7C) cells with 80 nM of MRGp resulted in either a slight inhibition or a slight stimulation on cell growth, respectively. When the cells were treated with MRGp and together with DHA, a significantly synergistic growth inhibition was observed. The growth of MDA-MB-436 cells was inhibited by 63% when the cells were treated with DHA and MRGp compared to 18% inhibition with DHA alone. Similarly, the growth of MDA-MB-468 cells was inhibited by 80% with DHA and MRGp compared to 22% inhibition with DHA alone.

### III. SUMMARY OF KEY DATA:

1. Expression of MRG protein was associated with human pregnancy-induced mammary gland differentiation.
2. Transfection of human breast cancer cells with MRG gene resulted in differentiated phenotypes.
3. Treatment of mouse whole mammary gland in organ culture with purified recombinant MRG protein induced gland differentiation with  $\beta$ -casein expression and differentiated morphology.
4. Transfection of breast cancer cells with MRG gene or treatment of the cells with MRG protein significantly enhanced DHA-induced growth inhibition.

### IV. SIGNIFICANCE:

1. There is an increasing public interest in the impact of pregnancy-induced differentiation on breast cancer incidence. As a hormonally related process, the evidence is now convincing, and it is widely accepted that early pregnancy and breastfeeding reduce the risk of breast cancer. Manipulation of pregnancy-like differentiation is a novel and broad approach for breast cancer prevention. Little is known about the regional and developmental expression of locally acting differentiating factors in the mammary epithelium during pregnancy. Within this content, a novel mammary derived growth inhibitor and a fatty acid binding protein (FABP) has recently been identified, characterized, and named Mammary derived growth inhibitor Related Gene (MRG). We now report that MRG, which is highly expressed in differentiated lactating human mammary gland, induces the functional differentiation of mammary epithelial cells. MRG is a candidate mediator of the differentiating effect of pregnancy and lactation on breast epithelial cells and up-regulation of MRG expression in young nulliparous females may mimic pregnancy- and lactation-induced mammary gland differentiation and prevent breast cancer incidence. MRG can also be used as a surrogate endpoint to guide for breast cancer prevention.
2. It is well established that omega-3 fatty acids DHA and EPA in fish oil have a suppressive effect on tumor growth and particularly on mammary tumorigenesis. Epidemiology studies, animal studies, and in vitro cell culture studies, support a role for  $\omega$ -3 fatty acids as adjunct therapy in prevention and treatment of breast cancer. Currently, the mechanisms by which DHA exerts its tumor suppressing activity remain controversial and unknown. As a new member in the family of FABP, MRG has the highest binding affinity to  $\omega$ -3 PUFA DHA. Our data, showing the differential inhibitory effects of DHA on different human breast cancer cells with or without MRG expression, suggest that the tumor suppressing activity of DHA on mammary gland may be mediated by MRG and presumably MRG-induced differentiation.



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- 18.

# Induction of Mammary Differentiation by Mammary-derived Growth Inhibitor-related Gene That Interacts with an $\omega$ -3 Fatty Acid on Growth Inhibition of Breast Cancer Cells<sup>1</sup>

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## ABSTRACT

We previously identified and characterized a novel tumor growth inhibitor and a fatty acid-binding protein in human mammary gland and named it the mammary-derived growth inhibitor-related gene (MRG). Here, the effects of MRG on mammary gland differentiation and its interaction with  $\omega$ -3 polyunsaturated fatty acids ( $\omega$ -3 PUFAs) on growth inhibition were investigated. MRG protein expression was associated with human mammary gland differentiation, with the highest expression observed in the differentiated alveolar mammary epithelial cells from the lactating gland. Overexpression of MRG in human breast cancer cells induced differentiation with changes in cellular morphology and a significant increase in the production of lipid droplets. Treatment of mouse mammary gland in organ culture with MRG protein resulted in a differentiated morphology and stimulation of  $\beta$ -casein expression. Treatment of human breast cancer cells with the  $\omega$ -3 PUFA docosahexaenoic acid resulted in a differential growth inhibition proportional to their MRG expression. MRG-transfected cells or MRG protein treated cells were much more sensitive to docosahexaenoic acid-induced growth inhibition than MRG-negative or untreated control cells. Our results suggest that MRG is a candidate mediator of the differentiating effect of pregnancy on breast epithelial cells and may play a major role in  $\omega$ -3 PUFA-mediated tumor suppression.

## INTRODUCTION

MRG<sup>3</sup> has been cloned in normal human mammary gland by differential cDNA sequencing aimed at the identification of growth inhibitory factors of the normal mammary gland (1). The sequence of MRG was found to be identical to the recently identified human B-FABP (Ref. 2). FABPs constitute a well-established family of cytoplasmic hydrophobic ligand-binding proteins and are thought to be involved in lipid metabolism by binding and transporting long-chain fatty acids intracellularly. However, other studies have implicated different roles for FABPs in cell signaling, growth inhibition, and differentiation (3-6). In particular, H-FABP, also known as MDGI, is abundantly expressed in differentiated lactating mammary gland and has been shown to inhibit growth of breast cancer cells (7-9). Among several subtypes of FABPs, only MRG/B-FABP and the previously identified H-FABP/MDGI have tumor-suppressing activity against breast cancer (2). These include the loss of MDGI (10) and MRG expression (1) during breast cancer progression, an inhibitory effect on proliferation of breast cancer cells (1, 7-10), and

suppression of breast tumor growth in the mammary fat pad nude mouse model (1, 11). In addition, the expression of both MRG (1) and MDGI (6) was mainly detected in myocardium, brain, and skeletal muscle, which are associated with an irreversibly postmitotic and terminally differentiated status of cells.

It is well established that  $\omega$ -3 PUFAs, primarily DHA and EPA in fish oil, suppress mammary tumorigenesis *in vivo* and breast cancer cell proliferation *in vitro* (12-21). As a member of FABP, it has been reported that  $\omega$ -3 PUFA DHA is the physiological ligand for mouse MRG (B-FABP), based on its high binding affinity ( $K_d = 10$  nM; Ref. 22). We have demonstrated that the gene encoding MRG has a strong tumor suppressor activity (1). The magnitude of the tumor-suppressing activity of MRG on mammary tumor is comparable to that observed previously for *Rb* and *p53* (23). In the current study, we investigated the effects of MRG on mammary differentiation and its interaction with DHA on the growth of breast cancer cells. Our data suggest that MRG is a differentiation factor for breast epithelial cells and that it may play a major role in DHA-mediated growth suppression of breast cancer cells.

## MATERIALS AND METHODS

**Cell Culture.** Human breast cancer cell lines MDA-MB-231, MDA-MB-436, and MDA-MB-468 were maintained in DMEM containing 5% FCS.

**Preparation of Anti-MRG Antibody.** A peptide sequence corresponding to amino acids 43-57 (1) was chosen for developing of the antibody because of its unique sequence for MRG. The peptide synthesis, purification, conjugation, and immunization of rabbits were conducted as we described previously (24). For final purification, a MRG peptide affinity column was made by conjugating 20 mg of MRG peptide to 5 ml of Aminolink resin (Pierce Chemical Co.), using sodium cyanoborohydride (Sigma).

**Immunohistochemical Staining.** As we described previously (1, 25), deparaffinized, rehydrated, and acid-treated human breast sections (5  $\mu$ m thick) were treated with H<sub>2</sub>O<sub>2</sub> and trypsin, and blocked with normal goat serum. Sections were incubated with a specific anti-MRG polyclonal antibody (1  $\mu$ g/ml) at 4°C overnight, followed by incubation with biotin-conjugated secondary antirabbit antibodies (DAKO). The colorimetric detection was performed using a standard indirect streptavidin-biotin immunoreaction method with DAKO's Universal LSAB Kit according to the manufacturer's instructions. There were some variations in staining intensity for MRG expression among the specimens. The negative cases were confirmed with at least two independent experiments. All stainings were reviewed by two pathologists.

**Preparation of MRGp.** The full-length MRG was amplified using standard PCR techniques with primers corresponding to the 5' and 3' sequences of the gene (5' primer, GGATCCCGTGGAGGCTTCTGT; 3' primer, GGTACCCCAGGGACATTTTA). The amplified fragment was gel-purified, and the DNA sequence was confirmed. As we described previously (24), a baculovirus expression vector, pA2-GP, was used to transform Sf9 cells. The purification of MRGp was performed as follows: (a) Medium supernatant, adjusted to pH 5.5, was first applied to tandem Poros HS/HQ columns (PerSeptive Biosystems) preequilibrated with 50 mM NaOAc (pH 5.5). (b) MRGp, collected in the flowthrough fraction, was adjusted to pH 8.0 and reapplied to the tandem Poros HS/HQ column preequilibrated with 20 mM Tris-HCl (pH 8.0). (c) MRGp, collected in the flowthrough fraction, was concentrated 50-fold, using a Filtron 3000 *M<sub>r</sub>* cutoff tangential-flow system and then separated on a Superdex-75 size-exclusion column equilibrated with 10 mM NaOAc (pH 6.5). (d) Pooled

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<sup>3</sup> The abbreviations used are: MRG, mammary-derived growth inhibitor-related gene; B-FABP, brain-type fatty acid-binding protein; H-FABP, heart-derived FABP; MDGI, mammary-derived growth inhibitor; PUFA, polyunsaturated fatty acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; MRGp, recombinant MRG protein.

MRGp fractions were applied to a hydroxyapatite column equilibrated with 10 mM NaOAc (pH 6.5); the weakly bound MRGp was eluted with 7.5 mM  $K_2HPO_4$  (pH 6.8). (e) MRGp fractions were then separated on a Superdex-75 size-exclusion column equilibrated with 65 mM  $Na_2HPO_4$ , 100 mM NaCl (pH 7.2). MRGp fractions were pooled and found to be >98% pure by SDS-PAGE with an endotoxin level <0.5 endotoxin units/mg. Purified MRGp was identified as a single band at 18 kDa in the SDS-PAGE by silver staining. The protein was analyzed for glycosylation by determining the monosaccharide content in a purified preparation, and the N-linked sugar chains were confirmed.

**Cell Morphology on Matrigel.** Cell morphology was determined using Matrigel-coated wells. Briefly, 6-well culture plates were coated with growth factor-reduced Matrigel (Collaborative Research) at 0.5 ml/well. Cells were then cultured in the coated wells with DMEM containing 5% fetal bovine serum. The cell morphology was observed under the microscope after 4 days.

**Detection of Cytoplasmic Lipids in Breast Cancer Cells.** Lipid accumulation was detected by oil red *O*-isopropanol staining as described previously (26). The cells were cultured on either Matrigel-coated plates or regular uncoated plates. After 4 days, the cells were fixed by 10% formaldehyde and subjected to oil red *O*-isopropanol staining. Accumulated lipids in the cells were stained red, and nuclei were stained blue by hematoxylin. Three independent observers counted the positive cells, and each observer randomly counted three fields ( $\times 40$ ). The numbers represent the average percentage of lipid accumulate cells from nine fields ( $\times 40$ ).

**Western Analysis.** Western blot analysis was conducted as we described previously (24). Briefly, the blot was incubated with anti-MRG primary antibody (1:800 dilution) overnight at 4°C, and then incubated with goat antirabbit IgG-horseradish peroxidase (1:6000 dilution) for 1 h, washed, and visualized by chemiluminescence.

**Mammary Gland Organ Culture.** Whole second thoracic mammary glands were removed from 7- and 10-week-old virgin female mice (FVB/n background) as described previously (27). The glands were cultured in medium 199 containing 5% FCS, with medium changed every 2 days. The medium was supplemented with following components from Clonetics: bovine pituitary extract (52  $\mu$ g/ml), insulin (5  $\mu$ g/ml), epidermal growth factor (10 ng/ml), and hydrocortisone (1  $\mu$ g/ml).

**In Vitro Assay for Cell Growth.** Cells were seeded in triplicate at 3000 cells/well (24-well plate) in 1 ml of DMEM-5% serum. For treatments with DHA or MRGp, cells were cultured in DMEM-1% serum. Cell growth was measured using the CellTiter 96 Aqueous Nonradioactive Cell Proliferation Assay Kit (Promega Corporation, Madison, WI).

**Statistical Analysis.** Values were expressed as means  $\pm$  SD. Statistical comparisons were made using the two-tailed Student's *t* test.

## RESULTS

**Association of MRG Expression with Mammary Gland Lactation.** In an attempt to evaluate the potential biological significance of MRG on the differentiation and lactation of the human mammary gland, we studied MRG protein expression in formalin-fixed, paraffin-embedded clinical human biopsy specimens from normal breast reduction mammoplasty specimens, lactating mammary glands, and malignant breast carcinomas.

Fig. 1 shows a representative immunohistochemical staining for MRG. The terminally differentiated lactating mammary gland is characterized by ducts branching into distended and large lipid-rich active secretory lobuloalveolar structures. An increase in cell volume as a

Fig. 1. Analysis of MRG protein expression on human breast tissues by immunohistochemical staining. Sections in panels A and B were stained with H&E with no immunohistochemical staining. Sections in panels C–E were stained immunohistochemically, with brown indicating MRG protein expression in mammary epithelial cells. All sections in C–E were also counterstained lightly with hematoxylin for viewing non-MRG-stained cells. A, epithelial cells in normal nonlactating lobules from a normal breast reduction mammoplasty specimen ( $\times 40$ ). B, epithelial cells in lactating lobules from a needle biopsy specimen ( $\times 40$ ). The differentiated lactating mammary epithelial cells have much diluted cytoplasm containing large lipid-rich secretory vacuoles (arrow). C, epithelial cells from lactating lobules showed very strong MRG staining ( $\times 10$ ). The specimen was from a 32-year-old lactating woman. The presence of vesicles containing milk protein (arrow) was noted. A serial slide from the same block was also incubated with nonimmunized control IgG, and no detectable background staining was observed at the same conditions as for the anti-MRG antibody. D, negative staining of normal lobular epithelial cells from a 25-year-old nulliparous woman with breast reduction mammoplasty ( $\times 10$ ). E, negative staining of MRG in a highly infiltrating breast carcinoma ( $\times 10$ ). A total of 23 clinical breast specimens were analyzed: 5 of 5 lactating samples were strongly positive; 10 of 10 infiltrating breast cancer samples were negative; 5 of 8 normal breast reduction mammoplasty samples were negative, and the remaining 3 normal breast samples were weakly positive.

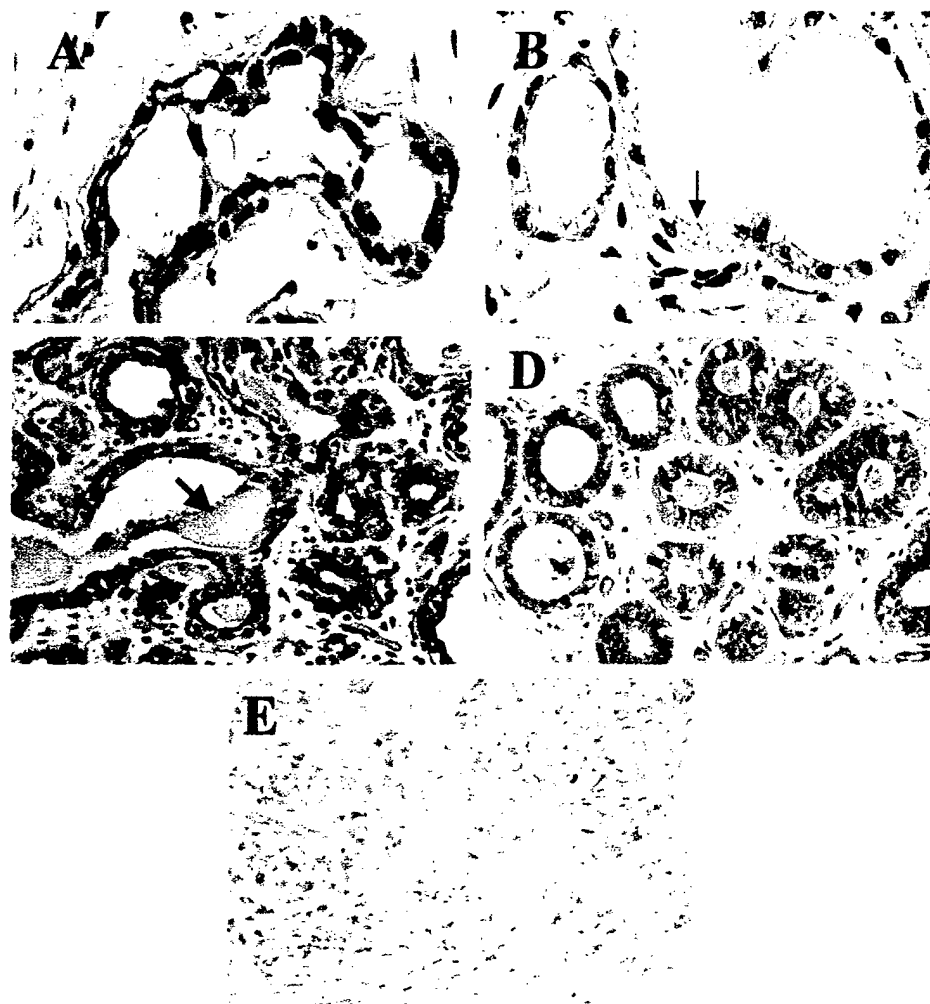




Fig. 2. Purity and immunoreactivity of the purified MRGp. A. SDS-PAGE of purified MRGp. Lane 1, molecular mass markers; Lane 2, MRGp (50 ng). The homogeneity of the purified MRGp was revealed by silver staining. B. Immunoblot with a specific anti-MRG antibody. The gel contained 30 ng of MRGp.

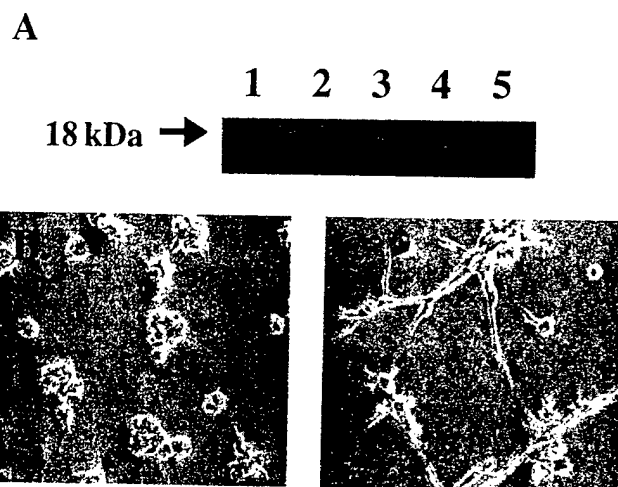


Fig. 3. Analysis of MRG expression and cell morphology. A. Western blot analysis of MRG protein expression. Total protein was isolated and normalized, and 25  $\mu$ g of total cellular protein were subjected to Western analysis with a specific MRG antibody. Lane 1, 60 ng of purified recombinant MRG protein; Lane 2, MRG-231-10; Lane 3, MRG-231-6; Lane 4, parental MDA-MB-231; Lane 5, neo-231-1. For morphology analysis, cells were culture on Matrigel-coated chamber slides for 6 days. B. MRG-231-10 cells were aggregated and formed spheroids. C. neo-231-1 cells had spreading morphology.

result of cytoplasmic vacuolation and the presence of secretory vesicles containing milk proteins was clearly noted in the lactating gland (Fig. 1B). We found strongly positive MRG protein staining in the alveolar mammary epithelial cells from the lactating mammary gland (Fig. 1C). The expression of MRG protein was clearly detectable in the alveolar epithelial cells in all five lactating mammary glands. In contrast, either no detectable MRG protein staining or very weak MRG protein expression was visualized in eight of the nonpregnant normal breast reduction mammoplasty specimens from nulliparous women (Fig. 1D). Expression of MRG protein was absent in all 10 cases of malignant breast carcinomas (Fig. 1E).

**Expression and Purification of MRGp.** Active MRGp is required to test its function on mammary epithelial cells. We expressed and purified MRGp prepared from baculovirus-infected Sf9 cells (see "Materials and Methods"). When analyzed by SDS-PAGE, the purified protein showed a single band at molecular mass of 18 kDa (Fig. 2A). The purified 18-kDa protein was confirmed as MRG by Western blot using a specific anti-MRG antibody (Fig. 2B).

**Induction of Differentiation of Breast Cancer Cells.** To investigate whether the high level of MRG expression in the lactating alveolar mammary epithelial is an instigator or merely a by-product of mammary gland differentiation leading to milk production, we investigated whether overexpression of the MRG gene could induce differentiation. We transfected MDA-MB-231 human breast cancer cells with full-length MRG cDNA and established several MRG-expressing clones (MRG-231 clones; Ref. 1). Fig. 3A shows the MRG protein

expression in MRG-231-10 and MRG-231-6 cells, two MRG-positive clones, but not in parental MDA-MB-231 and neo-231-1 MRG-negative cells.

It is well established that the extracellular matrix is required for normal functional differentiation of mammary epithelia. Striking changes in cell morphology were observed when MRG-231 cells were cultured in the Matrigel-coated dish. MRG-231-10 cells were aggregated to form spheroids on a reconstituted basement membrane gel (Fig. 3B), a typical differentiated phenotype for mammary epithelial cells (28). In contrast, neo-231-1 cells showed considerable heterogeneity in cell size, and many cells had "fibroblast-like" spreading morphology (Fig. 3C).

We examined whether MRG-induced morphological changes are consistent with differentiation. Because the maturation of breast cells is characterized by the presence of lipid droplets that are milk components, we examined lipid accumulation in MRG-231 cells compared with the control cells. Droplets containing neutral lipid were readily detectable in MRG-231-6 clones cultured in the uncoated culture plates; in contrast, no obvious lipid droplet could be observed in the neo-231-1 cells. When the lipid-producing cells were counted, 2 and 5% of MRG-231-6 and MRG-231-10 cells, respectively, produced lipid droplets, but virtually no lipid-producing cells were observed in MDA-MB-231 and neo-231-1 cells. When the cells were cultured in the Matrigel-coated plates, a significant increase in lipid accumulation was observed in both MRG-231 cells and MRG-negative control cells. Representative samples of lipid staining in MRG-231-6 and neo-231-1 cells are shown in Fig. 4. Fifteen percent of MRG-231-6 and 21% of MRG-231-10 cells produced lipid droplets, but only 4% of MDA-MB-231 cells and 3% of neo-231-1 contained lipid droplets, which were much smaller in size than those of MRG-positive cells (Table 1).

**Induction of Differentiation of Mouse Mammary Gland by MRGp.** Tissue-specific expression of milk protein in mammary epithelial cells depends on contact with stromal cells and matrix proteins. To further confirm the differentiating effect of MRG on mammary gland, we used whole-organ culture of mouse mammary glands to study whether MRGp can regulate milk protein  $\beta$ -casein. The

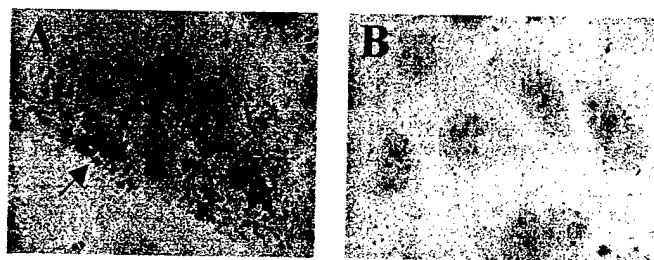


Fig. 4. Stimulation of lipid accumulation by MRG. Cells were cultured on Matrigel-coated dishes for 4 days. A, a representative field for MRG-231-10 cells ( $\times 40$ ). B, a representative field for neo-231-1 cells ( $\times 40$ ). Darker areas indicate lipid staining.

Table 1 Effects of MRG on the lipid accumulation of MDA-MB-231 cells

Cells were cultured either on Matrigel-coated plates or uncoated plates for 4 days, fixed, and subjected to oil red O-isopropanol staining. All slides were also counterstained lightly with hematoxylin for viewing nuclei. The positive cells were counted randomly in three fields ( $\times 40$ ), with each field containing 150 cells. Three observers counted a total of 1350 cells. The numbers represent the average percentage  $\pm$  SE of lipid accumulated cells from nine fields.

Cell lines	Lipid droplets in uncoated dish, lipid-producing cells/total (%)	Lipid droplets in Matrigel-coated dish, lipid-producing cells/total (%)
MDA-MB-231	0.2 $\pm$ 0.02	4 $\pm$ 0.9
neo-231-1	0.08 $\pm$ 0.01	3 $\pm$ 0.8
MRG-231-6	2 $\pm$ 0.4	15 $\pm$ 3.2
MRG-231-10	6 $\pm$ 1.8	24 $\pm$ 4

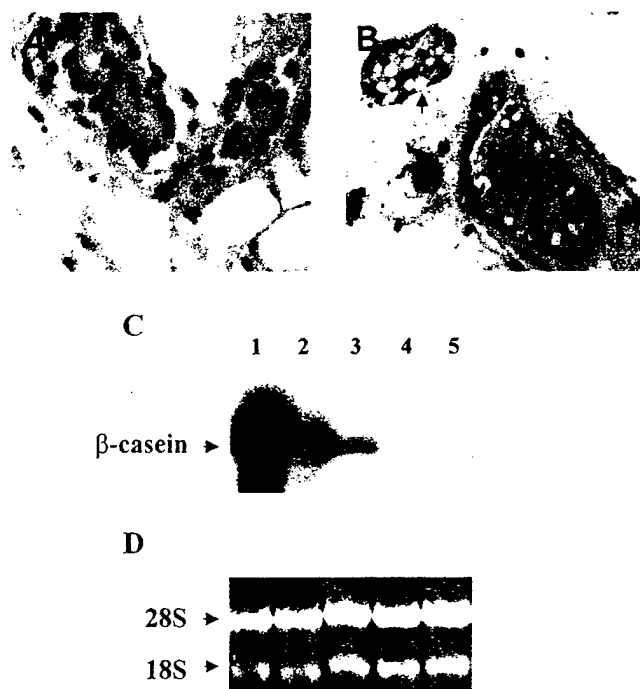


Fig. 5. Effects of MRGp on mammary gland morphology and  $\beta$ -casein expression. Second pairs of mouse whole thoracic mammary glands were cultured for 6 days with or without 50 nM MRGp in medium supplemented with bovine pituitary extract, insulin, epidermal growth factor, and hydrocortisone as described in "Materials and Methods." Fresh medium containing MRGp was added every 2 days. Half of the gland was subjected to fixing, sectioning, and histological analysis (A and B), and the other half was subjected to RNA extraction for Northern analysis of  $\beta$ -casein expression (C and D). Mammary gland histological analysis: A, control ( $\times 20$ ); B, MRGp-treated ( $\times 20$ ). The fat droplets accumulated in MRGp-treated alveolar epithelial cells were observed (arrows). Expression of  $\beta$ -casein mRNA (C) was analyzed by Northern blot and normalized by visualization of ribosomal bands (D). Lane 1, mammary gland from pregnant mouse as a positive control for  $\beta$ -casein; Lanes 2 and 3, MRGp-treated mammary glands in organ culture; Lanes 4 and 5, control untreated glands in organ culture. Mammary glands in Lanes 2 and 4 were derived from a 10-week-old virgin mouse; mammary glands in Lanes 3 and 5 were derived from a 7-week-old virgin mouse.

glands from virgin mice were cultured for 6 days with or without 50 nM MRGp. In mammary gland development, the alveolar buds represent a developmental pathway that eventually leads to secretory alveoli during functional differentiation. Histological examination of MRGp-treated glands revealed the appearance of secretory active alveoli with enlarged luminal spaces and the induction of lipid accumulation (Fig. 5, A and B). Consistent with these changes, which are characteristic for the differentiated phenotype, functional differentiation with stimulation of  $\beta$ -casein was also observed. Although no detectable  $\beta$ -casein mRNA was observed in control mammary glands, expression of  $\beta$ -casein mRNA was significantly increased in MRGp-treated glands (Fig. 5, C and D). Therefore, treatment of mouse mammary gland in organ culture with MRGp resulted in a histologically differentiated phenotype as well as functional differentiation.

**Interaction of the  $\omega$ -3 PUFA DHA and MRG on Cell Growth.** Because MRG is a fatty acid-binding protein with the highest binding affinity to the  $\omega$ -3 PUFA DHA, we were interested in studying whether the growth-suppressing effect of DHA is mediated in part by MRG. We first studied the effects of DHA on MRG-negative MDA-MB-231 cells. The cells were treated with DHA at doses of 2, 4, 6, 8, and 12  $\mu$ g/ml for 4 days, with fresh DHA added every 2 days. A very narrow dose-dependent growth inhibition was observed for DHA (Fig. 6A). Although no significant growth inhibition was observed for DHA at a dose of 2  $\mu$ g/ml, 71 and 92% growth inhibition was observed at doses of 8 and 12  $\mu$ g/ml, respectively. We therefore chose the non-inhibiting DHA dose of 2  $\mu$ g/ml to test its growth-regulatory effect on

MRG-positive versus MRG-negative cells. As demonstrated in Fig. 6B, when the cells were treated with 2  $\mu$ g/ml DHA, 55 and 47% growth inhibition was observed in MRG-231-6 and MRG-231-10 MRG-transfected cells, respectively. However, no growth inhibition was observed in MRG-negative parental MDA-MB-231 cells and neo-231-1 cells. We also studied the effect of the  $\omega$ -6 fatty acid linoleic acid on the growth of MDA-MB-231 cells. At the same conditions as for the  $\omega$ -3 fatty acid DHA, no significant growth effect was observed at the similar dose range between 4 to 20  $\mu$ g/ml (data not shown).

To further confirm the synergistic interaction of MRG expression and DHA on growth inhibition, we treated MRG-negative MDA-MB-436 and MDA-MB-468 cells with DHA and MRGp. MRGp treatment induced dose-dependent growth inhibition in MDA-MB-436 breast cancer cells (Fig. 7A). Although no significant growth inhibition was observed when the MRGp dose was  $<50$  nM, 10 and 14% growth inhibition was observed when cells were treated with 50 and 80 nM MRGp, respectively. At 150 nM MRGp, growth was inhibited 58%. A submaximal MRG dose of 80 nM was used to test the interaction between MRG and DHA. Treatment of MDA-MB-436 (Fig. 7B) and MDA-MB-468 (Fig. 7C) cells with 80 nM MRGp resulted in either a slight inhibition or a slight stimulation of cell growth, respectively. When the cells were treated with MRGp together with DHA, a significant synergistic growth inhibition was observed. The growth of MDA-MB-436 cells was inhibited by 63% when the cells were treated with DHA and MRGp, compared with 18% inhibition with DHA alone. Similarly, the growth of MDA-MB-468 cells was inhibited by 80% with DHA and MRGp, compared with 22% inhibition with DHA alone.

## DISCUSSION

MRG, identified and cloned by a differential cDNA sequencing approach as a novel human breast cancer growth inhibitor (1), has

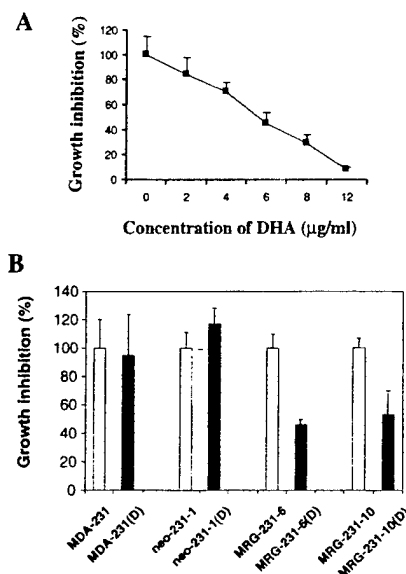


Fig. 6. Differential growth inhibition by DHA on MRG-positive and MRG-negative breast cancer cells. Cells were cultured in DMEM containing 1% FCS and treated with DHA at different concentrations for 4 days. Medium containing fresh DHA was added every 2 days. Cell growth was measured as described in "Materials and Methods." A, dose-response curve of DHA on MDA-MB-231 cells. B, effect of DHA on MRG-positive and -negative cells. The cells were treated (filled columns) or not treated (open columns) with 2  $\mu$ g/ml DHA. All values were normalized to the percentage of untreated control cells, which was taken as 100%. The numbers in both A and B represent the means of three cultures; bars, SE.

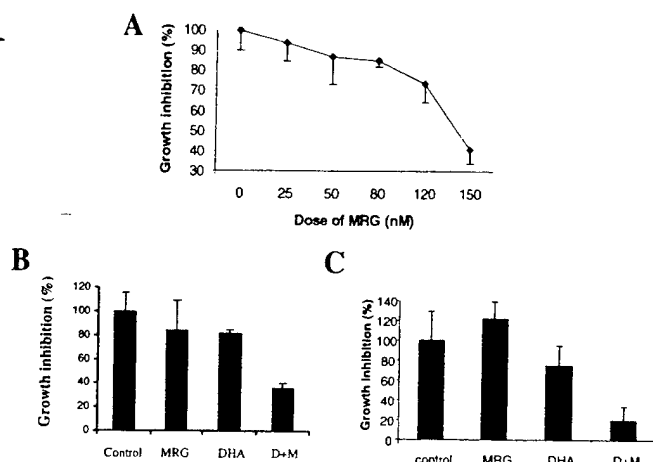


Fig. 7. Synergistic effects of MRGp and DHA on growth inhibition. All cells were cultured in DMEM containing 1% FCS. A, MDA-MB-436 cells were cultured with different doses of MRG for 4 days. MDA-MB-436 (B) and MDA-MB-468 (C) cells were treated with 80 nM MRGp, 2  $\mu$ g/ml DHA, or MRGp plus DHA (D + M) for 4 days. Medium containing fresh MRGp and DHA was added every 2 days. All values were normalized to the percentage of untreated control cells, which was taken as 100%. The numbers in both represent the means of three cultures; bars, SE. Statistical comparisons for both cell lines treated with DHA and MRGp relative to the cells treated with DHA alone indicated  $P < 0.001$  for growth inhibition.

sequence identical to that of the recently identified B-FABP (2). MRG/B-FABP has no sequence homology to any of the hitherto known growth inhibitors. The exact function of B-FABP has not been identified. Cellular fatty acid-binding proteins are a highly conserved family of proteins involved in intracellular fatty acid metabolism and trafficking. It has been suggested that in brain and heart, B-FABP and H-FABP regulate the supply of fatty acids to the mitochondria for  $\beta$ -oxidation (29, 30). The mammary gland, however, is a highly lipogenic tissue, and fatty acids are not likely to be a major fuel for its metabolism. Therefore, MRG/B-FABP and MDGI/H-FABP could fulfill different functions in mammary gland compared with brain and heart. We demonstrated that (a) MRG expression was associated with human mammary gland differentiation, with the highest expression in the terminally differentiated alveolar mammary epithelial cells from the lactating gland, and (b) that MRG induced differentiation of mammary epithelial cells.

MRG protein expression was undetectable in breast carcinomas by immunohistochemical staining, which is consistent with the previous *in situ* hybridization data on the loss of MRG transcription in breast carcinomas. Although in the previous *in situ* hybridization analysis, MRG transcripts could be detected in the epithelial cells from normal mammary glands (1), in the current immunohistochemical analysis of MRG protein expression, MRG protein staining was either very weak or undetectable in nondifferentiated mammary glands from nulliparous women. This discrepancy may reflect the different sensitivities of the more sensitive *in situ* hybridization versus the less sensitive immunohistochemical staining. Alternatively, the tested different normal breast specimens may represent different stages of differentiation. It is also possible that this discrepancy between the *in situ* hybridization and immunohistochemical staining is attributable to the fact that the message may not be translated. Nevertheless, addition of MRGp to cultures of breast cancer cells and to organ cultures of mouse mammary gland induced growth inhibition and gland differentiation. Although the mechanism for cellular uptake of MRGp is not clear, it is likely that MRGp diffuses through the membrane because of its very hydrophobic and lipogenic nature. In fact, some FABPs such as H-FABP (MDGI) can be secreted and detected in milk (9).

In addition to the differentiating effect on mammary gland, the

expression of MRG also correlates with neuronal differentiation in many parts of the mouse central nervous system (31, 32). Furthermore, blocking antibody for MRG/B-FABP can block glial cell differentiation (31). MDGI/H-FABP protein has been detected mainly in myocardium, skeletal and smooth muscle fibers, lipid and steroid-synthesizing cells of adrenals, lactating mammary gland, and terminally differentiated epithelia of the respiratory, intestinal, and urogenital tracts (6). The results provide evidence that expression of MDGI is associated with an irreversibly postmitotic and terminally differentiated status of cells. Therefore, it seems clear that a differentiation-associated function is a common property of this structurally related subfamily of FABPs.

It is well established that the  $\omega$ -3 fatty acids DHA and EPA, found in fish oil, have a suppressive effect on tumor growth and particularly on mammary tumorigenesis. Epidemiological studies (33–37) support a role for  $\omega$ -3 fatty acids as adjunct therapy in the prevention and treatment of breast cancer. This protective effect of  $\omega$ -3 PUFAs can be demonstrated in animal models with carcinogen-induced mammary tumors in mouse and rat and mammary xenografts in nude mice (14–19). Various mechanisms have been proposed to explain the tumor-suppressive activity of  $\omega$ -3 PUFAs; of special interest are alteration of the oxidative metabolism of arachidonic acid via the cyclooxygenase pathway (35) and changes in lipoxygenase activity (reviewed in Ref. 36). Lipid peroxidation, the oxidation of long-chain PUFAs, can produce an array of secondary products of lipid oxidation that may possess cytostatic or cytolytic capacity. It has been proposed that DHA and EPA can both directly and indirectly modulate gene expression (38). The direct effects of DHA and EPA are most probably mediated by their ability to bind to positive and/or negative regulatory transcription factors, whereas the indirect effects appear to be mediated through alterations in the generation of intracellular lipid second messengers.

At present, the mechanisms by which DHA exerts its tumor suppressing activity remain controversial and unknown. As a newly identified fatty acid-binding protein and a growth differentiation factor for mammary cells, we have demonstrated here that treatment of human breast cancer cells with DHA resulted in differential growth inhibition proportional to the MRG expression in the cells: MRG-positive cells or MRGp-treated cells were much more sensitive to DHA-induced growth inhibition than MRG-negative cells or control, untreated cells. Our data suggest that the growth-suppressing activity of DHA on breast cancer cells may be mediated in part by MRG and presumably by MRG-induced differentiation. This hypothesis is also supported by a previous report that DHA has the highest binding affinity for mouse B-FABP (MRG), suggesting that the physiological ligand for MRG is the  $\omega$ -3 PUFA DHA (22).

The impact of pregnancy and lactation on breast cancer risk recently has been of great interest in terms of breast cancer prevention. As hormonally related processes, it is widely accepted that pregnancy at an early age and breastfeeding reduce the risk of breast cancer (39–42). The possibility of preventing breast cancer by manipulation of these processes with hormones or dietary factors such as  $\omega$ -3 PUFAs that mimic the differentiating effect is a novel and manipulable approach to breast cancer intervention and prevention. However, little is known about the regional and developmental expression of locally acting growth factors and differentiating factors in the mammary epithelium during pregnancy and lactation. Within this context, MRG could play a role in both mammary gland differentiation and  $\omega$ -3 PUFA-mediated antitumor effect. The potential application of MRG as a biomarker for mammary gland differentiation to assess the efficiency of differentiation-based breast cancer chemoprevention and to predict tumor-suppressive response to  $\omega$ -3 PUFAs warrants further investigation.

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